In Situ Quantification of Biological N₂ Production Using Naturally Occurring \(^{15}\text{N}\)\(^{15}\text{N}\)

Laurence Y. Yeung,†∥ Joshua A. Haslun,‡ Nathaniel E. Ostrom,‡ Tao Sun,† Edward D. Young,§ Maartje A. H. J. van Kessel,‖ Sebastian Lücke,‖ and Mike S. M. Jetten‖

†Department of Earth, Environmental and Planetary Sciences, Rice University, Houston, Texas 77005, United States
‡Department of Integrative Biology and Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, Michigan 48824, United States
§Department of Earth, Planetary, and Space Sciences, University of California-Los Angeles, Los Angeles, California 90095, United States
‖Department of Microbiology, Radboud University, Nijmegen 6525 AJ, The Netherlands

Supporting Information

ABSTRACT: We describe an approach for determining biological N₂ production in soils based on the proportions of naturally occurring \(^{15}\text{N}\)\(^{15}\text{N}\) in N₂. Laboratory incubation experiments reveal that biological N₂ production, whether by denitrification or anaerobic ammonia oxidation, yields proportions of \(^{15}\text{N}\)\(^{15}\text{N}\) in N₂ that are within 1\%\text{e} of that predicted for a random distribution of \(^{15}\text{N}\) and \(^{14}\text{N}\) atoms. This relatively invariant isotopic signature contrasts with that of the atmosphere, which has \(^{15}\text{N}\)\(^{15}\text{N}\) proportions in excess of the random distribution by 19.1 ± 0.1\%\text{e}. Depth profiles of gases in agricultural soils from the Kellogg Biological Station Long-Term Ecological Research site show biological N₂ accumulation that accounts for up to 1.6% of the soil N₂. One-dimensional reaction-diffusion modeling of these soil profiles suggests that subsurface N₂ pulses leading to surface emission rates as low as 0.3 mmol N₂ m\(^{-2}\) d\(^{-1}\) can be detected with current analytical precision, decoupled from N₂O production.

INTRODUCTION

Biological N₂ production constitutes the main mechanism through which fixed nitrogen is returned to the atmosphere. While many methods have been developed for measuring N₂ production in the field, obtaining accurate estimates of ecosystem fixed-nitrogen loss remains a challenge. Field-based techniques often require nutrient amendments (e.g., \(^{15}\text{N}\)-labeled nitrate), manipulation of biochemical pathways (e.g., C\(_2\)H\(_4\) inhibition of nitrous oxide reductase),\(^3\) or sampling and incubation of soil core samples, all of which introduce poorly constrained uncertainties. For example, in nutrient amendment studies, the fraction of extant nitrogen substrate utilized must be accounted for, but it is often difficult to constrain. Moreover, biological N₂ production can be stimulated by substrate addition, biasing measurements based on this approach. Soil-core incubations to evaluate N₂ production may not require nutrient amendments, but instead require that the extant gases be replaced with a gas mixture to reduce or replace the ambient N₂ background. Ultimately, this suite of methods for quantifying N₂ production rates can only probe short-term and potential rates of denitrification and other nitrogen-loss processes. Importantly, they may not integrate variation in activity that occurs over longer time scales at a given sampling site. Passive in situ measurements are rare, and fraught with a different set of complications: a recent attempt to use N₂/Ar ratios to probe excess N₂ production in situ found that physical fractionation of gases, combined with insufficient sensitivity, would likely preclude its widespread application.

Stable isotopes of nitrogen at natural abundance levels could in principle be used to determine the amount of biologically produced N₂ in soil gases as well. Variations in the \(^{15}\text{N}/^{14}\text{N}\) ratio of N₂, reported as a \(\delta\)-value in per mil (\%\text{e}) relative to atmospheric N₂,

\[
\delta^{15}\text{N} = \frac{^{15}\text{N}}{^{14}\text{N}} \times 10^3 - 1
\]

\[
^{15}\text{R} = \frac{^{15}\text{N}}{^{14}\text{N}}
\]

can be caused by variability in the chemistry of N₂ cycling, substrate \(\delta^{15}\text{N}\), and physical transport. Nevertheless, a large isotopic contrast may exist between biological and atmospheric...
N₂: strong isotopic fractionation for N₂-yielding processes can result in local deviations in the δ¹⁵N value of N₂ relative to their substrates and the atmospheric background. However, closed-system and rate-dependent effects on isotopic fractionation, the broad distribution of substrate δ¹⁵N values, and physical fractionation affecting the elemental and isotopic composition of soil gases are rarely well-characterized, rendering the interpretation of bulk δ¹⁵N values in soil N₂ non-unique; disentangling the variations in δ¹⁵N of soil-N₂ may not be possible without additional constraints.

We recently developed methods to measure δ¹⁵N in N₂ with high precision at natural abundances, which offers a new approach to quantifying in N₂ production on local and global scales. Together with δ¹⁵N/δ¹⁴N ratios, measurements of the δ¹⁵N/¹⁵N¹⁴N ratio in N₂ yield a “clumped” isotope tracer, Δ₃₀₀, which is defined below and also reported in per mil:

\[
\Delta_{300} \equiv \frac{30^0 R_{\text{sample}}}{30^0 R_{\text{random}}} - 1 \tag{3}
\]

\[
30^0 R_{\text{sample}} = \frac{15N^{15}N}{14N^{14}N} \tag{4}
\]

\[
30^0 R_{\text{random}} = \left(\frac{15N}{14N}\right)^2 \tag{5}
\]

Unlike the δ¹⁵N value, Δ₃₀₀ represents the proportional (rather than absolute) enrichment in δ¹⁵N/δ¹⁴N, quantified relative to a random distribution of ¹⁵N and ¹⁴N atoms in N₂ molecules. The δ¹⁵N value of the substrate does not affect the Δ₃₀₀ signature of a N₂-yielding process because the Δ₃₀₀ value is normalized against the bulk ¹⁵N/¹⁴N ratio (eqs 3 and 5). Moreover, the Δ₃₀₀ tracer is insensitive to physical fractionation and nitrogen fixation; these processes tend to preserve proportions of ¹⁵N¹⁵N relative to ¹⁴N¹⁴N. Furthermore, the Δ₃₀₀ values of the biological N₂ thus far identified cluster near zero, while the Δ₃₀₀ value of atmospheric N₂ is 19.1 ± 0.1‰—a signature of upper-atmospheric gas-phase reactions. It results in a large isotopic contrast between biological and atmospheric N₂. Local subatmospheric Δ₃₀₀ values in soils thus may reflect the presence of biological N₂, which can be quantified through a clumped-isotope mass balance if the Δ₃₀₀ signatures of different N₂-yielding pathways are sufficiently similar. Δ₃₀₀ values may trace biological N₂ production in situ using the same principles first laid out by Hauck and co-workers, but without the need for nutrient amendments or isotopic labels.

Motivated by this potential application, we conducted a broader survey of Δ₃₀₀ values from biological processes. Specifically, we expanded our earlier characterization of Δ₃₀₀ values from denitrifying bacteria with new measurements of Δ₃₀₀ signatures from anaerobic ammonia-oxidizing (anammox) bacteria and incubations of natural soils. The narrow distribution of biological Δ₃₀₀ signatures that we find suggests that Δ₃₀₀ values can indeed be used to quantify biological N₂ production in soils, and possibly also other restricted environments. As a proof-of-principle application, we present two soil-gas depth profiles that show evidence for biological N₂ production, and evaluate the sensitivity of the approach.

### EXPERIMENTAL METHODS

Isotopic analyses were performed on the ultrahigh resolution Nu Instruments Panorama mass spectrometer at the University of California, Los Angeles according to methods described previously. The uniquely high resolution of the instrument allows the simultaneous measurement of δ¹⁴N/²⁸N and δ¹⁵N/²⁷N ratios at m/z = 29 and 30, with near-baseline resolution of ¹⁴N¹⁴O from ¹³C¹⁸O and ¹²C¹⁶O at m/z = 30. N₂ gas samples (20–50 μmol) were isolated from experimental headspace and soil-derived gases using cryogenic purification on a high-vacuum sample preparation line followed by gas chromatographic separation from O₂ and Ar before isotopic analysis. Cryogenic purification removes condensable gases (e.g., CO₂ and some hydrocarbons) and was accomplished by passing the gas through a stainless-steel U-trap submerged in liquid nitrogen (~196 °C). The gas was then condensed onto silica gel pellets at ~196 °C within the sample-injection loop of the gas-chromatographic system. N₂ gas was separated from O₂ and Ar using a molecular sieve SA column (3 m × 1/8” OD) followed by a HayeSep D column (2 m × 1/8 in. OD) inline, all with a 20 mL min⁻¹ He flow rate at 25 °C. The sample gases, air, and high-temperature standards of N₂ (which were heated at 800 °C for 24–48 h over strontium nitrate) were purified the same way and analyzed during the same analytical sessions. Analytical precision for replicate air samples during these sessions was ±0.006‰ for δ¹⁵N and ±0.08‰ for Δ₃₀₀.

To determine the Δ₃₀₀ signatures of N₂ produced by anammox bacteria, headspace outflows from several anammox bioreactors at Radboud University were sampled. Outflows from bioreactors containing enrichment cultures of the genera Candidatus Kuenenia and Ca. Brocadia (both freshwater genera), as well as Ca. Scalindua (a marine genus) were sampled using a 8 mL sampling loop made of 1/4 in. OD stainless steel tube. The gas mixture was transferred cryogenically to a pre-evacuated sample finger filled with silica gel at ~196 °C for 15 min before flame-sealing. All enrichment cultures at Radboud University were grown on the same NH₄SO₄ + NaN₃ substrates, which had δ¹⁵N values of 0.5 ± 0.3‰ and 26.2 ± 0.3‰, respectively. Atmospheric contamination was monitored using gas chromatography–mass spectrometry of the outflow, using O₂ (m/z = 32) as a proxy. A correction for air-N₂ contamination in the bioreactor headspace was calculated from the O₂ signal and a proportionality coefficient determined through a series of volumetrically calibrated mixtures of air in the 95% Ar/5% CO₂ mixtures used to flush the bioreactors. Measured air contamination varied between bioreactors, ranging from 0.6% for Kuenenia to 12.3% for Scalindua outflows, as a result of variable anammox activity compared to the flushing flow rate. After correction for background contamination (0.12–2.40‰ for δ¹⁵N and 0.1–2.3‰ for Δ₃₀₀), duplicate collections showed reproducibility in δ¹⁵N and Δ₃₀₀ within ±0.01‰ and ±0.3‰, respectively.

Incubations of natural soils were performed to determine the Δ₃₀₀ signatures of N₂ produced by natural biological communities. Soils from three agricultural treatments at the Kellogg Biological Station (KBS) Long-Term Ecological Research site were used for these experiments. Soils at the site belong to the Kalamazoo series, which are fine-loamy, mixed mesic Typic Hapludalfs. Soils T1 and T2 are mixed mesic Typic Hapludalfs. T1 and T2 are from an early successional old field community (containing grasses, shrubs, and trees) that was established in 1989 and has been maintained by an annual spring burn since 1997. Incubations of 25-g soil samples were conducted in 125 mL glass serum bottles that were crimp-sealed using butyl...
rubber stoppers (Geomicrobial Technologies, Inc., Ochelata, OK, U.S.A.). Initially, after saturating the dried soils, an anaerobic headspace was created by sparging with He. The soils were then allowed to denitrify for 7–10 d to remove any initial oxidized N. At that point, the headspace was sparged again with He and then inoculated with glucose (0.3 mL, 1 M) and NaNO₃ substrate (1 mL, 0.3 M; δ¹⁵N = 5.4‰). Production of N₂ was allowed to proceed for 96 h to ensure collection of sufficient N₂ gas for isotopic analysis. Gases were transferred cryogenically to a pre-evacuated silica-gel finger and flame-sealed prior to analysis at UCLA.

For the in situ study, soil gas samples from the KBS Interactions Experiment site were obtained from a monolith soil lysimeter. The lysimeter is located 5 m from the edge of the KBS T2 (no-till agricultural) KNO₃ 0.1 and T1 (conventional agricultural) KNO₃ 0.1 × 1.22 m × 2.03 m (L × W × D) monolith lysimeter was installed with a minimum of disturbance to the soil column approximately 5 cm above the soil surface in 1986 as described in Brown et al.²³ Gas sampling lines (stainless steel, 1.6 mm OD, 0.5 mm ID) were previously installed through the walls of the lysimeter and extend 30 cm outward. Each line was purged by removing 3 mL of soil gas (~50 times the line volume) by gastight syringe and discarding the gas. Subsequently, 5 mL of gas for each sample was collected by gastight syringe and pushed through a 3 mL stainless-steel sampling bottle that had been previously purged with He gas. Gas samples were collected on 10/11/17 and 7/18/18 at depths of 24, 34, 50, 59, 77, 86, and 170 cm from the soil surface. On return to the laboratory, gases were cryogenically purified and transferred to a pre-evacuated silica-gel finger and flame-sealed.

### RESULTS AND DISCUSSION

#### Δ₃₀ Values from Biological N₂ Production Are Near Zero. Anammox enrichment cultures produced N₂ with Δ₃₀ values close to, but slightly different from the stochastic distribution of isotopes (Table 1). Nitrogen gas produced by the two freshwater genera are characterized by Δ₃₀ < 0 (i.e., N₂ was “antclumped”), while N₂ produced by the marine Ca. Scalindua enrichment had Δ₃₀ = 1.0 ± 0.3‰e, indistinguishable from an equilibrium distribution of ¹⁵N isotopes at culturing temperatures (i.e., 1.0‰e at 35 °C). A positive correlation between Δ₃₀ and δ¹⁵N values was observed when all anammox culture data are considered together (R² = 0.86, p = 0.0009).

The origins of this correlation were not investigated, but deserve further scrutiny: the apparent difference in Δ₃₀ value between freshwater and marine species may point to a different biochemistry related to the gene organization and subsequent expression of hydrdine synthase enzyme.²⁴,²⁵ In any case, the Δ₃₀ values for N₂ produced by freshwater anammox genera are close to that expected from combinatorial isotope effects:²⁶ the contrast in isotopic compositions between the NaNO₂ (δ¹⁵N = −26.2‰) and NH₄SO₄ (δ¹⁵N = −0.5‰) substrates, by itself, would yield Δ₃₀ = −0.2‰e, close to the mean measured values of −0.2 ± 0.1‰e and −0.5 ± 0.5‰e (±σ) for Ca. Kuenenia and Ca. Brocadia, respectively. Isotopic fractionation during biological uptake may cause additional variability in the δ¹⁵N value of the assimilated substrates, but the Δ₃₀ value of the N₂ produced is not expected to deviate more than ~1‰e from zero because the combinatorial effect is a relatively weak function of the substrate δ¹⁵N contrast.²⁶

Anaerobic incubation of KBS soils yielded N₂ with Δ₃₀ values indistinguishable from the stochastic distribution of isotopes (i.e., all within 0.2‰e; see Table 1). Unlike in previous axenic laboratory cultures of denitrifying bacteria,¹⁴ no statistically significant dependence on reaction extent or δ¹⁵N values was observed (p = 0.2–0.4 for a slope of zero, depending on the soil; see Table S1 of the Supporting Information, SI).

Compiling these results with those from earlier experiments on bacterial denitrifiers¹⁴ shows that biological N₂ production yields Δ₃₀ values between −0.7‰e and +1.4‰e, with a weak dependence, if any, on bulk δ¹⁵N values (Table S1). Moreover, the lack of Δ₃₀ fractionation during biological nitrogen fixation¹³ suggests that it preserves Δ₃₀ values in the N₂ residue. Atmospheric N₂, in contrast, is characterized by δ¹⁵N values indistinguishable from the stochastic distribution of δ¹⁵N values (i.e., all within 0.2‰).⁵¹ Due to the large and relatively invariant δ¹⁵N contrast in isotopic compositions between the NaNO₂ (δ¹⁵N = −26.2‰) and NH₄SO₄ (δ¹⁵N = −0.5‰) substrates, by itself, would yield Δ₃₀ = −0.2‰e, close to the mean measured values of −0.2 ± 0.1‰e and −0.5 ± 0.5‰e (±σ) for Ca. Kuenenia and Ca. Brocadia, respectively. Isotopic fractionation during biological uptake may cause additional variability in the δ¹⁵N value of the assimilated substrates, but the Δ₃₀ value of the N₂ produced is not expected to deviate more than ~1‰e from zero because the combinatorial effect is a relatively weak function of the substrate δ¹⁵N contrast.²⁶

### Table 1. Clumped-Isotope Composition of N₂ (±1σ) Derived from Experimental Cultures of Denitrifying or Anammox Bacteria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Δ₃₀ (‰e)</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural soils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KBS T1 (conventional agricultural)</td>
<td>KNO₃</td>
<td>−0.1 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>KBS T2 (no-till agricultural)</td>
<td>KNO₃</td>
<td>0.1 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>KBS T7 (early successional)</td>
<td>KNO₃</td>
<td>0.2 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>Anammox enrichment cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kuenenia spp.</td>
<td>NH₄SO₄ + NaNO₂</td>
<td>−0.2 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>Brocadia spp.</td>
<td>NH₄SO₄ + NaNO₂</td>
<td>−0.5 ± 0.3</td>
<td>2</td>
</tr>
<tr>
<td>Scalindua spp.</td>
<td>NH₄SO₄ + NaNO₂</td>
<td>1.0 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>Denitrifying bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>KNO₃</td>
<td>0.9 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>Paracoccus denitrificans</td>
<td>KNO₃</td>
<td>0.6 ± 0.2</td>
<td>5</td>
</tr>
</tbody>
</table>
While the soil-gas $^{28}R_{\text{soil}}$ and $^{30}R_{\text{soil}}$ values can be measured (as $^{15}N$ and $^{30}N$ soil values) and $^{28}R_{\text{atm}}$ and $^{30}R_{\text{atm}}$ are known, this system of equations remains under-constrained. However, the proportionality between $^{28}R_{\text{bio}}$ and $^{30}R_{\text{bio}}$ coming from a relatively invariant biological clumped-isotope signature ($\Delta_{\text{bio}}$) provides a way forward.

Two-component mixing is linear in $\Delta_{30}$ values if the biologically produced $N_2$ has the same $^{15}N/14N$ ratio as that of the atmosphere, i.e., $\delta^{15}N_{\text{bio}} = \delta^{15}N_{\text{atm}}$ yielding eqs 8 and 9:

$$\Delta_{30,\text{soil}} = (1 - f_{\text{bio}})\Delta_{30,\text{atm}} + f_{\text{bio}}\Delta_{30,\text{bio}}$$

(8)

In that case, the soil-gas $\Delta_{30}$ value ($\Delta_{30,\text{soil}}$) would be simply related to $f_{\text{bio}}$ and the atmospheric ($\Delta_{30,\text{atm}}$) and biological clumped-isotope signatures. Measurements of $\Delta_{30,\text{soil}}$ would allow one to solve for $f_{\text{bio}}$:

$$f_{\text{bio}} = \frac{\Delta_{30,\text{atm}} - \Delta_{30,\text{soil}}}{\Delta_{30,\text{atm}} - \Delta_{30,\text{bio}}}$$

(9)

Unknown and variable $\delta^{15}N_{\text{bio}}$ values lead to deviations from this relationship, and uncertainty in $f_{\text{bio}}$. However, for $\Delta_{30,\text{soil}}$ values close to $\Delta_{30,\text{atm}}$ (i.e., mixtures dominated by atmospheric $N_2$), eqs 8 and 9 retain much of their accuracy over a wide range of $\Delta^{15}N_{\text{bio}}$ values (Figure 2). For example, when $\delta^{15}N_{\text{bio}}$ is 20% different from $\delta^{15}N_{\text{atm}}$, the $f_{\text{bio}}$ value derived from eq 9 is within 6% of the true $f_{\text{bio}}$ value (e.g., a calculated $f_{\text{bio}}$ of 0.094 when the true $f_{\text{bio}}$ is 0.1). The expected range of $\Delta_{30,\text{bio}}$ values coming from natural communities of $\pm 1\%$—i.e., the range observed in laboratory experiments—results in an additional $\pm 6\%$ relative uncertainty in $f_{\text{bio}}$ (e.g., an error of $\pm 0.006$ for $f_{\text{bio}} = 0.1$). Both errors are similar to that contributed by analytical uncertainty for $f_{\text{bio}} = 0.1$ (resulting in a cumulative uncertainty of $\pm 10\%$ if added in quadrature), but they quickly decrease in importance as $f_{\text{bio}}$ decreases: for $f_{\text{bio}} = 0.01$, analytical uncertainty of $\pm 0.08\%$ in $\Delta_{30}$ results in an asymmetrical uncertainty of $+36\%$ and $-56\% f_{\text{bio}}$ i.e., $f_{\text{bio}} = 0.010^{+0.036}_{-0.006}$. Therefore, analytical uncertainty dominates $\Delta_{30}$-based estimates of $f_{\text{bio}}$ for $f_{\text{bio}} < 0.1$. Current analytical uncertainties suggest that soil gas containing $\geq 1\%$ biological $N_2$ will be detectable in $\Delta_{30,\text{soil}}$ values.

To test this concept, we obtained two depth profiles of $\delta^{15}N$ and $\Delta_{30}$ values in $N_2$, along with $N_2O$ concentrations, from a monolith lysimeter installed in the KBS Interactions site. We found that many $\Delta_{30,\text{soil}}$ values were less than or equal to $\Delta_{30,\text{atm}}$ (Figure 3 and Table S2), ranging from 18.8‰ to 19.1‰. One sample analysis (34 cm depth on 10/11/17) was rejected based on apparent contamination that resulted in an abnormally elevated $\Delta_{30}$ value ($4\sigma$ above the mean atmospheric value measured during the analytical session). The largest $\Delta_{30,\text{soil}}$ depletions ($-0.3 \pm 0.1\%$ relative to $\Delta_{30,\text{atm}}$), observed in both profiles, correspond to $1.6\%-0.5\%$ of soil $N_2$ at those depths being derived from biological processes. Soil-$N_2$ $\delta^{15}N$ values were equal to or slightly lower than the atmospheric value, although they differed between profiles: the profile obtained in July 2018 had $\delta^{15}N$ values close to the atmospheric value, while the profile obtained in October 2017 had sub-atmospheric $\delta^{15}N$ values ranging from $-0.4$ to $-0.6\%$. $N_2O$ concentrations increased nearly monotonically with increasing depth, with values exceeding 1000 parts per billion (ppb) at 170 cm depth (Figure 4). Taken together, these data imply an active nitrogen cycle and the presence of biological $N_2$ in these soils.

**Gas Diffusion and Denitrification Hot-Spots Can Explain Observed Soil $\Delta_{30}$ Profiles.** A further understanding of the chemical and isotopic signatures measured in the soil gas can be obtained using a one-dimensional diffusion-reaction model based on Fick’s second law:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial z^2} + J(z, t)$$

(10)

where $D$ is the effective gas diffusivity and $J(z, t)$ is the production rate of a gas, which may be depth-($z$) and time-($t$) dependent. We treat the soil-gas system as a diffusive column ventilated to the atmosphere at the top ($z = 0$) and with zero permeability at the bottom ($z = 180$ cm). At steady state ($\partial C/\partial t = 0$) the depth profile is described by $\partial^2 C/\partial z^2 = -J/D$; because $J$ and $D$ are positive as defined, concentration depth profiles at steady state should monotonically decrease toward the atmospheric value. Isotopic tracers may increase or
decrease toward the top depending how they are defined, but the change with depth should be monotonic toward the atmospheric value.

The depth profiles are not in steady state with respect to N₂. At steady state, deeper soil-gas would have accumulated low-Δ₃₀ biological signals over time, resulting in Δ₃₀ values increasing from depth to the surface. The N₂O depth profiles show accumulation at depth, but the N₂ profiles do not (Figures 3 and 4). Instead, Δ₃₀ values are close to atmospheric values at depth, decrease at mid-depths, and return to atmospheric values at the surface. Pulsed biological N₂ production over a limited depth range is required to reproduce these mid-depth minima in Δ₃₀ values. Specifically, a quiescent period with respect to N₂ production, which ventilates the soil down to 170 cm, must precede the pulse. Quantitative ventilation is not necessary, however; the quiescent period need only be long enough to dilute remnant Δ₃₀ signals from earlier events beyond the limits of detection (~5 days for the expected diffusivities; see below). Denitrification “hot moments” related to heterogeneities in soil moisture and organic carbon availability have the appropriate temporal and spatial variability. The contrast between N₂ and N₂O depth profiles suggest that their production during these hot moments can be temporally decoupled. Moreover, the accumulation of N₂O at depth argues against ventilation via gas exchange at the lysimeter–soil interface as the origin of the nonsteady-state Δ₃₀ depth profile.

The shapes of the Δ₃₀ depth profiles can be reproduced by solving eq 10 using a 0.1-day N₂ production pulse of Gaussian shape (1 cm full width at half-maximum), followed

### Table 2. Model Parameters Used to Derive Profiles in Figure 3

<table>
<thead>
<tr>
<th>sampling date</th>
<th>center depth (cm)</th>
<th>pulse peak (nmol N₂ cm⁻³ s⁻¹)</th>
<th>sampling lag (h)</th>
<th>N₂ production (mmol N₂ m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/11/17</td>
<td>59</td>
<td>1.2</td>
<td>1.9</td>
<td>5.7</td>
</tr>
<tr>
<td>7/18/18</td>
<td>37</td>
<td>2.6</td>
<td>26.4</td>
<td>12.9</td>
</tr>
</tbody>
</table>

*Pulses are Gaussian (1 cm full width at half maximum), occurring for 0.1 days.
by a small time lag between the N₂ pulse and sampling (see Table 2). Here, we assume an air-filled porosity, \( \epsilon \), of 0.05—resulting in a calculated \[^{10,31}\] soil diffusivity of \( D_s = 0.0039 \text{ cm}^2 \text{s}^{-1} \) for N₂—and \( \Delta_{30,\text{bio}} = 0 \). The assumed \( \epsilon \) value is within the plausible range for these soils \[^{32}\] so it is appropriate for illustrative purposes. Using these parameters, the modeled \( \Delta_{30,\text{soil}} \) depth profile for July 2018 (the best-fit curve using a least-squares algorithm) reflects a depth-integrated gross production of 10.4 mmol N₂ m\(^{-2} \) remaining in the soil after a 12.9 mmol N₂ m\(^{-2} \) pulse (Figure 3A). The \( \delta^{15} \text{N} \) values of N₂ in that profile can be reproduced if the biological N₂ has \( \delta^{15} \text{N} = -11\%_e \), on average. Note that the particular pulse shape, duration, and sampling lag used here (Table 2) is likely one of many that can explain the data and therefore not meant to be diagnostic; consequently, the profile is considered a local (rather than global) best fit. The depth-integrated gross production, however, should be robust for a given air-filled porosity. For example, the model can also yield a satisfactory fit of the data using a 10-fold longer initial N₂ pulse length of 1 day with a correspondingly weaker peak pulse peak of 0.3 mmol N₂ cm\(^{-3} \) s\(^{-1} \) (instead of 2.6 mmol N₂ cm\(^{-3} \) s\(^{-1} \)). Both scenarios yield scaled-up N₂ pulse magnitudes (3–4 kg N ha\(^{-1} \)) that are consistent with peak N₂ fluxes observed in previous in lab\[^{33}\] and field\[^{35}\] experiments.

The modeled \( \Delta_{30} \) depth profile for October 2017 shown in Figure 3 implies a depth-integrated gross production of 5.7 mmol N₂ m\(^{-2} \) using a pulse centered at 59 cm (Figure 3B, Table 2). Unlike for the July 2018 profile, the \( \delta^{15} \text{N} \) values of N₂ in that profile cannot be explained by biological N₂ production alone. Gravitational fractionation over this depth range would increase \( \delta^{15} \text{N} \) values by \( <0.01\%_e \), so other physical mechanisms such as diffusive fractionation and/or water vapor flux fractionation \[^{13}\] may be especially important for this profile. Sampling took place the morning after a heavy overnight precipitation event (>40 mm), implicating a physical isotope effect such as a hydrologically driven diffusive influx of atmospheric N₂. These physical mechanisms will not affect \( \Delta_{30,\text{soil}} \) values significantly because they fractionate proportionately over a small \( \delta^{15} \text{N} \) range. In addition, solubility fractionation does not seem to affect clumped-isotope compositions of sparingly soluble gases \[^{14,35}\], despite its effects on both elemental \[^{36}\] and bulk-isotope composition. Consequently, the \( \Delta_{30} \) tracer shows a clearer measure of biological N₂ production than the \( \delta^{15} \text{N} \) value of N₂.

If these biological N₂ pulses are isolated in time, then equivalent surface N₂ fluxes \( \mathcal{F} \) can be derived from the reaction-diffusion models, and the results compared to previous measurements of KBS soils. For one-dimensional diffusion, the equation \( \mathcal{F} = [N_{2,\text{bio}}] \times D_s / \epsilon \) describes the instantaneous surface gas flux, where \( [N_{2,\text{bio}}] \) is the concentration of biological N₂ and \( \epsilon \) is the depth from the surface. For \( \epsilon = 5 \text{ cm} \), the biological N₂ flux from the top 5 cm of soil, are shown in Figure 5. The flux \( \mathcal{F} \) for the two profiles ranges from 0.1–2.9 mmol N₂ m\(^{-2} \) d\(^{-1} \) (3–81 mg N m\(^{-2} \) d\(^{-1} \)) during the first 10 days after the pulse events, with a prolonged period of low, but nonzero flux lasting several times longer (e.g., \( \mathcal{F} = 0.1–0.2 \) mmol N₂ m\(^{-2} \) d\(^{-1} \) for the 7/18/18 profile between 10 and 20 days after the pulse). These estimates are comparable to previous amendment-stimulated N₂ production rates from these soils. In particular, Bergsma et al. (2001) reported surface N₂ fluxes of 0.2–2.0 mmol N₂ m\(^{-2} \) d\(^{-1} \) (6–55 mg N m\(^{-2} \) d\(^{-1} \)) during a four-day experiment utilizing a surface flux chamber and an amendment of \(^{15} \text{N} \)-labeled KNO\(_3\). \[^{38}\] The model-derived fluxes strongly depend on the assumed air-filled porosity \( \epsilon \)—which was not measured directly and can vary in time and space—so this agreement may be coincidental. Nevertheless, the two methods appear to yield results on the same order of magnitude. More well constrained in situ soil-atmosphere fluxes can be obtained with concurrent measurements of soil physical properties.

The only comparable in situ method for quantifying biological N₂ production in soils is the N₂/Ar method. Yang and Silver (2012) reported a relatively high detection limit of 3.9 mmol N₂ m\(^{-2} \) d\(^{-1} \) for surface-flux measurements, \[^{12}\] larger than the calculated peak surface fluxes shown in Figure 5. While the method can analytically resolve N₂ excesses of less than 0.1%, \[^{40}\] physical fractionation of N₂ and Ar in soils presents substantial systematic uncertainties in these environments. We hypothesize that measurements of N₂/Ar soil profiles may yield limited improvements in uncertainty because the physical mechanisms complicating the interpretation of \( \delta^{15} \text{N} \) values of N₂ (e.g., the water vapor flux fractionation) \[^{13}\] fractionate N₂/Ar ratios to a greater degree, offsetting any analytical sensitivity advantages. Soil \( \Delta_{30,\text{soil}} \) depth profiles, in contrast, are insensitive to physical fractionation, revealing evidence for biological N₂ production in soil profiles despite the lower analytical sensitivity of the method. N₂ fluxes into the atmosphere can be derived from \( \Delta_{30,\text{soil}} \) profiles if soil physical properties (i.e., air-filled porosity and diffusivity) are determined independently. The method could be used to compare in situ production rates to incubation- and amendment-based methods in field studies, or to obtain independent estimates using an array of spatially dispersed observations across soil types and conditions. Time series of soil-gas profiles similar to those shown here, sampled through
lysimeters or air-permeable tubing, would provide a long-term perspective on soil $\text{N}_2$ production dynamics, which is presently difficult to access without perturbing soil biogeochemistry and is useful for models. Analytical throughput (2–3 samples/day) and availability of instrumentation are currently limiting factors for the $\Delta_{30}$ approach, but the relatively long ventilation time scales of certain soils may still allow weekly to-monthly sampling to capture the impacts of hot moments.

The initial results reported here suggest that $\Delta_{30,\text{end}}$ signals are sufficiently large that the approach can be used in future assessments of site- and ecosystem-scale loss of fixed nitrogen. Furthermore, the approach can also be applied to marine environments to investigate both the magnitude and mechanisms of fixed-nitrogen loss in low-oxygen zones. Finally, constraining biological $\text{N}_2$ production globally using $\Delta_{30,\text{atm}}$ appears possible in principle if the terms related to upper-atmospheric chemistry in the global $\Delta_{30}$ budget—both the isotopic reordering rates and $\Delta_{30}$ endmembers—can be refined.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b00812.

Compilation of isotopic composition data for $\text{N}_2$ produced during pure- and enrichment-culture experiments reported here and in ref 14; isotopic composition data for $\text{N}_2$ and concentrations of $\text{N}_2\text{O}$ in soil gases (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: lyeung@rice.edu.*

**ORCID**

Laurence Y. Yeung: 0000-0001-9901-2607

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This research was supported by the U.S. National Science Foundation grant EAR-1349182 to L.Y.Y. and E.D.Y., EAR-1348935 to N.E.O., the David and Lucile Packard Foundation grant EAR-1349182 to L.Y. and E.D.Y., EAR-1640609 to M.S.M.J., and NSF Long-term Ecological Research Grant DEB-1637653 at the Kellogg Biological Station and by Michigan State University AgBioResearch.

We would like to thank Guylaine Nuijten for her contributions to maintaining the anammox bioreactors at Radboud University, Kevin Kahmark and Stacey Vanderwulp for their assistance with monolith lysimeter sampling, Reinhard Well for discussions, and four anonymous reviewers and the editor for comments that improved this work.

**REFERENCES**


(15) Yeung, L. Y.; Young, E. D.; Schaeuble, E. A. Measurements of $^{15}\text{O}$/$^1\text{O}$ and $^{17}\text{O}$/$^1\text{O}$ in the atmosphere and the influence of isotope-exchange reactions. *J. Geophys. Res.* 2012, 117, D18306.


